Environment & Ecology 27 (3A) : 1369—1373, 2009
 ISSN 0970-0420

Isolation and Genetic Characterization of an Endosulfan Degrading Bacterium *Bacillus stearothermophilus*

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Abstract

A bacterium having ability to degrade endosulfan was isolated from pesticide contaminated soil and identified. Morphological and biochemical characteristics of the organism confirmed it as *Bacillus stearothermophilus.* The organism was grown in a medium containing 0.3% endosulfan as a sole source of sulfur. The plasmid curing of *Bacillus stearothermophilus* using ethidium bromide revealed the plasmid born nature of the organism. Endosulfan-degradation was a sulfur-starvation response of this bacterium. The plasmid transformed *E. coli* BL-21strain has grown only at 0.07% of endosulfan concentration in non-sulphur medium.
Key words : *Bacillus stearothermophilus,* Characterization, Endosulfan, Plasmid curing.

Endosulfan is an organochlorine group of pesticide, emerged as a leading chemical against broad spectrum of insects and mites. It is used extensively throughout the world to control the insect pests of a wide range of crops including cereals, tea, coffee, cotton, fruits, oil seeds and vegetables. It is not recommended for household use. Intentional misuse of endosulfan for killing fish and anails has been reported. The technical endosulfan is a mixture of two isomers, viz. α-endosulfan and β-endosulfan in the ratio 7:3. Technical grade endosulfan contains 94% α-endosulfan and β-endosulfan and other related compounds like endosulfan alcohol, endosulfan ether and endosulfan sulfate. US Environmental Protection Agency (EPA) classifies endosulfan as highly hazardous. World Health Organization (WHO) classified as moderately hazardous (Romeo and Quijano 2000).

It was earlier considered as safer alternative to other organochlorine pesticides in many countries. But in the last two decades, because of its abundant usage and potential transport, endosulfan contamination is frequently found in the environment at considerable distances from the point of its original applications (Miles and Pfeuffer 1997). Endosulfan has been detected in the atmosphere, soils, sediments, surface and rainwater and food stuffs. It is extremely toxic to fish and aquatic invertebrates (Sunderam et al. 1992). It has been implicated in mammalian gonadal toxicity, genotoxicity and also neurotoxicity (Chaudhuri et al.1999).

Microbial degradation of pesticide may play an important role in detoxifying the pesticide contaminated sites in the environment. Accelerated biodegradation of soil-applied pesticides upon their repeated application is the result of soil-pesticide-microbe interaction and can undermine the efficacy of the pesticides under consideration (Sethunathan 1971).Biodegradation ability in microorganisms is achieved through isolation of pesticide degrading genes (Sutherland et al. 2002). Many microorganisms have been investigated as a source of xenobiotic degrading enzymes (Javed et al. 2001). In the present study, the endosulfan degrading bacterium was isolated and identified as *B. stearothermophilus* and its plasmid borne character was confirmed.

Methods

Isolation and Characterization

Fertile topsoil was obtained from grape field belonging to division of Horticulture University of Agriculture Sciences Bangalore, Karnataka. This field received several applications of endosulfan from past five years. Soil was enriched for endosulfan degrading micro-organisms by treating with endosulfan. The bacterial population in the endosulfan treated samples was estimated by serial dilution plate method. Endosulfan was added to the nutrient agar so as to obtain concentrations 0.07, 0.15, 0.22, 0.30 and 0.37%. From the serially diluted solution, 1 ml suspension of each 10^{-5} dilution was separately added to the sterile petri plates. Then, the endosulfan treated nutrient medium was poured into the petri plates and allowed for solidification. These plates were incubated for a week at room temperature and total bacterial population was estimated. Predominant colonies growing at higher concentration of endosulfan treated medium from the soil sample was transferred to fresh medium and purified by repeated streaking on nutrient agar medium treated with 0.30% endosulfan. This pure culture was used for characterization. The isolate was further inoculated on non-sulfur-medium treated with different concentrations of endosulfan and inoculated at room temperature for 5 days. Observation for growth was recorded. The bacterium was characterized by following the taxonomic key delineated in Prokaryotes (Balows et al. 1991) and consulting the Bergey's manual for the characters (Krieg and Holt 1986). The bacterial isolate was identified as *Bacillus stearothermophilus* based on morphological, cultural and biochemical characters.

Isolation of Plasmid DNA

The plasmid DNA was isolated by following the method of Sambrook et al. 1993. The plasmid DNA was treated with DNAse free RNAse for 1 hour at 65C. After 1 h the DNA sample was taken out and stored for further use at 4C. Estimation of the molecular weight of the intact plasmid was done by comparison with standard DNA. The standard used was 0.07—12.7 kb DNA ladder.

Plasmid Curing

Plasmid curing is the removal of the plasmid from bacterial cell. Plasmid curing was done to find out plasmid born nature to confirm the location of gene responsible for the degradation of endosulfan. To the petriplates, non-sulfur medium (NSM) containing 0.30% of endosulfan was added along with ethidium bromide (0.01% wt/vol). The culture was streaked and incubated at 37C for 2 days. The grown bacterium was repeatedly sub-cultured until no growth of the bacterium was noticed.

Preparation of Competent Cells using Calcium Chloride Method

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. Bacteria which are able to uptake DNA are called "competent" and are made so by treatment with calcium chloride in the early log phase of growth. The procedure followed was as mentioned by Dagert and Ehrlich (1979).

Bacterial Transformation

Eppendorf tube containing 200 µl competent cells was taken out of -20C and kept at 4 c for thawing. After the competent cells become semi-fluid, tubes were taken to inoculation hood and plasmid DNA was added to the competent cells tube and kept at 4 C for transformation. The cells were heat shocked at 42 C for 90 seconds and immediately kept on ice. Then 800 µl LB was added to the mixture and incubated for 90 minutes at 37C. The cells were pelleted by centifuging and majority of the supermatant was poured off and 40 µl of the supernatant was retained. The cells were resuspended in LB medium and plated them on selection medium.

Growth of E. coli BL-21 on NSM Treated with Different Concentration of Endosulfan

The bacterial cells were grown on selection medium (non-sulfur-medium) containing 0.07 to 0.37% endosulfan. One plate was kept as control containing NSM without endosulfan. The growth of bacterium was noticed and observation was recorded after two days.

Results and Discussion

Effect of Different Concentrations of endosulfan on Bacterial Population

The population for endosulfan resistant bacteria was analyzed for soil sample and the observations recorded are presented in Table 1. The soil sample

Table 1. Effect of different concentrations of endosulfan on bacterial population. The data represent an average of three replications. - = Absence of growth, CFU = Colony forming unit.

Endosulfan concentration (%)	Population of bacteria (× 10^5 CFU/g soil)
0.07	15.2
0.15	8.1
0.22	6.4
0.30	5.0
0.37	

collected was inoculated to the nutrient medium treated with different concentrations of endosulfan. More bacterial population was observed at lower concentration of endosulfan. As the endosulfan concentration in the medium increases, the bacterium population decreased and there was no growth of bacterium at 0.37%. This indicated that the 0.37% endosulfan was fatal to soil bacteria. This also indicates that the insecticide can inhibit bacteria at higher concentrations.

A bacterium growing at higher concentrations of endosulfan was selected and purified for further study. This bacterium was inoculated on to non-sulfur medium with or without endosulfan. The isolates did not grow on sulfur free agar medium (Table 2). But, it has grown on sulfur free medium supplemented with different concentrations of endosulfan indicating that the organism can use endosulfan as a source of sulfur. Microorganisms as source of many enzymes, could degrade different xenobiotic compounds. The ability of degradation depends on their genetic make up for the enzyme. Jilani and Khan (2004) isolated a *Pseudomonas* strain capable of degrading pesticides malathion, cypermethrin and captan. They noticed higher growth of *Pseudomonas* population at lower concentration of pesticides. However, higher concentrations inhibited the growth of the bacterium. These results are in agreement with the works of Sutherland et al. (2002) who isolated *Mycobacterium* strain ESD by enrichment culture technique using endosulfate as a sole source of sulfur.

Table 2. Growth of the bacterial isolate on non-sulfur medium containing different concentration of endosulfan. The data represent an average of three replications. + = Presence of growth, – = Absence of growth.

Endosulfan concentration (%)	Growth of isolate
0.0	–
0.07	+
0.15	+
0.22	+
0.30	+
0.37	–

Table 3. Characters of the *Bacillus stearothermophilus* grown at higher concentration of endosulfan.

Characters	Descriptions
Morphological Characters	
Color of the colony	White
Colonial morphology	Irregular/filamentous flat colonies
Oxygen requirement	Aerobic
Cell shape	Rods
Biochemical Characters	
Gram reaction	Gram positive
Catalase activity	Positive
Endospore	Positive
MR/VP test	Negative
Growth at 65 C	Growth present at 65 C
Growth on non-sulfur medium (NSM)	No growth

Isolation and Characterization of Endosulfan Degrading Bacterium

The bacterium was characterized by following the taxonomic key delineated in Prokaryotes (Balows et al. 1991) and consulting the Bergey's manual for the characters (Krieg and Hoet 1986). The bacterial isolate was identified as *Bacillus stearothermophilus* based on morphological, cultural and biochemical characters (Table 3). The other important character of *Bacillus stearothermophilus* is the growth at 65 C. In the present study, the organism has grown at 65 C. However, the organism did not grow at 75 C temperature (data not shown). The ability of the bacteria to grow at higher temperature may be due to the rate of reactivation of denatured thermophilic α-amylase, which is rapid between 40-50 C and maximum at 55C, though the organism could grow up to 65 C (Ogasahara et al. 1970). All these characters confirmed it as *B. stearothermophilus.*

Table 4. Growth of *Bacillus stearothermophilus* during different generations of plasmid curing. +++ - Good growth, ++ - Moderate growth, + - Decreased growth, – - No growth.

No of generations	Growth of *Bacillus stearothermophilus* on NSM treated with endosulfan (0.30%) and ethidium bromide (0.01%)
I	+++
II	+++
III	++
IV	++
V	+
VI	—

Plasmid Curing of Bacillus stearothermophilus

The plasmids are extracellular, circular DNA molecules harboring in bacterial cells. The plasmid DNA was isolated and subjected to electrophoresis. The plasmids of *Bacillus stearothermophilus* had a molecular size of ~12.7 Kb (Mielenz 1983). The treatment of cells with a substance that interferes with plasmid replication is the agent responsible for plasmid curing and the process is called as plasmid curing. In the present study ethidium bromide was used as plasmid curing agent. Ethidium bromide (0.1%) was added to the NSM medium treated with 0.30% of endosulfan and *Bacillus stearothermophilus* culture was grown for six generations with repeated subculture. The *Bacillus stearothermophilus* growth was observed up to five generations after repeated subculture on NSM medium having endosulfan (0.30%) and ethidium bromide (0.1%). This showed that the plasmid has been removed by curing and the endosulfan degrading gene is a plasmid borne. Sally and Rosenblum (1971) reported that the ethidium bromide is an efficient curing agent for the penicillinase plasmids of *Staphylococcus aureus* strains. Stephen and Baldwin (1972) reported 96.1 to 100% loss of the ability to produce penicillinase by two strains of *Staphylococcus aureus* (PC1 and 196E), grown on medium containing 0.002% sodium dodecyl sulfate (SDS). Crameri et al. (1986) also used the DNA-intercalating agent ethidium bromide to eliminate plasmid DNA from Streptomycetes (Table 4).

Plasmid Mediated Degradation of Endosulfan

Becterial cells host different types of plasmids like, fertility plasmids, resistant plasmids, cole plasmids, degradative plasmids and virulence plasmids. Among these, degradative plasmids are responsible for degradation of organic and inorganic molecules. The results revealed the genes responsible for endosulfan degradation are present on the plasmid DNA as the bacterium was unable to grow in presence of endosulfan after plasmid curing (Table 4). Friedrich et al. (1983) also isolated *Pseudomonas oxalaticus* borne Plasmid pJP4 encoding the ability to degrade the herbicide 2, 4-dichloro phenoxy acetic acid. Javed et al. (2001) reported a novel bacterium *Bacillus* sp. harboring a megaplasmid of about 60kb responsible for utilization of DMP as the sole source of carbon. Bhadbhade et al. (2002) reported *Pseudomonas mendocina* degrading monocrotophos, by 67% and harbored a 7.4 kb plasmid, designated as pMCP424.

Table 5. Growth of plasmid transformed *Escherichia coli* BL 21 on endosulfan treated non-sulfur medium. + = Growth present, – = Growth absent

Endosulfan concentration (%)	Growth of transformed *E. coli* BL 21 from *Bacillus stearothermophilus*
0.07	+
0.15	–
0.22	–
0.30	–
0.37	–

Transformation of E. coli BL 21 with Bacillus stearothermophilus

The pesticide degrading plasmid present in *Bacillus stearothermophilus* was transferred to the *E. coli* strain BL-21. The advantage of using *E. coli* strain BL-21 is that strain is free from native plasmid and the expression of foreign gene/plasmid can be easily identified in the system. In the present study, the plasmid isolated from *B. stearothermophilus* was transformed to *E. coli* strain BL-21 and grown on different concentrations of endosulfan treated medium. The plasmid transformed *E. coli* has grown at 0.07% endosulfan in the medium (Table 5). This is further indicated as the plasmid borne character of the bacterium to degrade endosulfan. However, the transformed *E. coli* fails to grow at higher concentration (0.15, 0.22 and 0.30%) of endosulfan. The can be attributed to the difference in the expression system of *E. coli* compared to *B. stearothermophilus.* Mielenz (1983) reported similar on expression of the thermostable alpha-amylase gene from the thermophilic bacterium *Bacillus stearothermophilus* in *Escherichia coli*. The present study confirmed that the *Bacillus stearothermophilus* is an endosulfan degrading bacterium and it can degrade endosulfan up to 0.3% in soil. The endosulfan degrading gene present in the plasmid and it could be transformed to *E. coli* for further characterization of the gene on the plasmid. Further studis are essential to characterize the gene present in this plasmid and use for plant transformation.

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